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# Cobalt and Chromium Ions Reduce Human Osteoblast-Like Cell Activity In Vitro, Reduce the OPG to RANKL Ratio, and Induce Oxidative Stress

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**ABSTRACT:** Metal-on-metal hip arthroplasty is associated with elevated levels of cobalt and chromium ions. The effects of cobalt and chromium ions on cell number, activity, expression of osteoprotegerin (OPG) and receptor activator of nuclear factor kappa B ligand (RANKL) and oxidative stress on human osteoblast-like cells were addressed. Saos-2 cells were supplemented with  $\text{Co}^{2+}$ ,  $\text{Cr}^{3+}$ , or  $\text{Co}^{2+} + \text{Cr}^{3+}$  (1:2) at 0, 1, 10, and 100  $\mu\text{g/L}$  and incubated for 24, 48, 72, and 96 h. Cell activity was assessed by MTT-assay and cell number by Crystal Violet staining. RNA levels of OPG and RANKL were evaluated using real-time quantitative polymerase chain reaction (qPCR). Compared to controls  $\text{Co}^{2+}$  reduced cell numbers: at 10  $\mu\text{g/L}$  by  $17 \pm 8\%$  after 48 h and at 100  $\mu\text{g/L}$  after 24 h by  $35 \pm 8\%$ .  $\text{Cr}^{3+}$  decreased cell numbers at 10  $\mu\text{g/L}$  after 48 and 72 h.  $\text{Co}^{2+} + \text{Cr}^{3+}$  combined at 1  $\mu\text{g/L}$  lowered cell numbers after 24 and 96 h ( $17 \pm 13$ , resp.  $13 \pm 4\%$ ). The 10 and 100  $\mu\text{g/L}$  concentrations reduced cell numbers significantly after 24, 48, and 96 h.  $\text{Cr}^{3+}$  reduced osteoblast activity at 1, 10, and 100  $\mu\text{g/L}$  at all incubation times. The strongest reduction ( $11 \pm 1\%$ ) was seen at 100  $\mu\text{g/L}$  after 96 h. The OPG/RANKL ratio was reduced after 72 h with almost all  $\text{Co}^{2+}$  and  $\text{Cr}^{3+}$  concentrations. After 96 h, glutathione, superoxide dismutase, and catalase levels were indicative for an oxidative stress response in all samples. In conclusion, cobalt and chromium ions reduce human osteoblast activity, reduce OPG/RANKL ratio and lead to oxidative stress. © 2011 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. *J Orthop Res* 30:740–747, 2012

**Keywords:** metal-on metal; osteoblasts; oxidative stress; OPG; RANKL

Total hip arthroplasty (THA) is a well-accepted therapy for hip osteoarthritis, with long lasting good clinical results. Survival rates of most THA's are documented at more than 95% over 10–15 years. Wear, osteolysis, and loosening have emerged as the dominant concerns among orthopedic hip surgeons. Polyethylene wear particles trigger a foreign body granulomatous reaction, inflammation, and immune reactions leading to the secretion of various bone resorbing agents (cytokines, prostaglandins). This results in peri-prosthetic bone loss, contributing to loosening and failure of the prosthesis.<sup>1</sup> One of the key factors in peri-prosthetic osteolysis is receptor activator of nuclear factor kappaB ligand (RANKL) and strong correlations have been found between its expression and the amount of polyethylene wear debris and the degree of osteolysis.<sup>2</sup> Furthermore, Masui et al.<sup>3</sup> also related the presence of polyethylene to RANKL expression. In general, osteoclastogenesis is largely regulated by receptor activator of nuclear factor kappaB (RANK), RANKL, and osteoprotegerin (OPG). RANKL stimulates differentiation of osteoclast precursor cells into mature osteoclasts and is required for osteoclast activity; in fact, RANKL-treated mice show increased bone resorption. On the other hand, OPG decreases the number of osteoclasts

and mice treated with OPG exhibit lowered osteoclast activity and increased bone volume.<sup>4</sup>

In order to avoid polyethylene wear-related loosening and the associated osteolytic problems, metal-on-metal articulation has become popular. Although lower volumetric wear has been shown in the laboratory, metal-on-metal articulation has been associated with higher serum and synovial levels of cobalt and chromium ions compared to metal-on-polyethylene THA articulation. The effects of these ions on bone formation and resorption are still subject to debate. Wang et al.<sup>1</sup> showed that cobalt and chromium ions are not toxic for osteoblasts in concentrations up to 100 ng/ml. Conversely, Fleury et al.<sup>5</sup> recently demonstrated the toxic effects of high concentrations of cobalt (0–10 ppm) and chromium (0–150 ppm) ions on MG-63 osteoblasts in terms of cell number and cellular activity. However, neither of these studies examined the effect of cobalt and chromium ions on the expression of RANKL and OPG by osteoblasts. Metal ions are also known to induce oxidative stress in tissues and cell cultures.<sup>6</sup> On the other hand, in research settings, cobalt ions can also be used as inducer of hypoxia and can induce apoptosis.<sup>7–12</sup>

The aim of the present study was to assess the temporal effect of cobalt and chromium ions on human osteoblast-like cells (Saos-2) in terms of cytotoxicity (cell number and cellular activity), the influence on the expression of bone turnover regulatory proteins RANKL and OPG, and on oxidative stress.

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## METHODS

### Cell Culture

Human osteoblast-like cells, originally isolated from a human osteosarcoma [Saos-2, obtained from the American Type Culture Collection (HTB-85)], were maintained in Dulbecco's modified Eagle's medium (DMEM-low glucose,) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Breda, NL), and 1% penicillin/streptomycin (Invitrogen), and were passaged every 3–5 days. Cells were cultured at 37°C in a humidified 5% CO<sub>2</sub>-atmosphere. At 70–80% confluence, adherent cells were detached using 0.25% trypsin ethylenediamine-1,2-diaminoethane solution (Trypsin EDTA, Sigma-Aldrich, Zwijndrecht, NL). For the experiments,  $1.5 \times 10^4$  cells were seeded in 1.5 ml culture medium in 2 cm<sup>2</sup> dishes (24-well plates).

### Cobalt and Chromium Ions

CoCl<sub>2</sub>·6H<sub>2</sub>O (Merck Nederland bv, Amsterdam, NL) and CrCl<sub>3</sub>·6H<sub>2</sub>O (Sigma-Aldrich) were added in such a way that three solutions at four different concentrations were created: cobalt ions at 0, 1, 10, and 100 µg/L, chromium at 0, 1, 10, and 100 µg/L, and cobalt–chromium (1:2) at 0, 1, 10, and 100 µg/L of culture medium. In the latter solutions, twice as much chromium ions as cobalt ions were added, as this reflects the clinical situation with metal-on-metal total hip arthroplasties, where experience suggests that blood chromium concentration is roughly double blood cobalt concentration (1.10, resp. 2.50 µg/L<sup>13</sup>). In addition, we found synovial Cr<sup>3+</sup> concentration to be more than twice the synovial Co<sup>2+</sup> concentration in a revision procedure of an uncemented metal-polyethylene total hip arthroplasty with documented wear and loosening (0.60, resp. 1.60 µg/L; personal data). The choice of 1, 10, and 100 µg/L was based on the median serum cobalt concentrations of 0.83 µg/L (maximum 15.6 µg/L) found in previous clinical work on cemented metal-on-metal total hip arthroplasties.<sup>1,14–16</sup> All solutions were filter-sterilized. Saos-2 cells were incubated with metal ion-containing culture medium in a humidified 5% CO<sub>2</sub>-atmosphere at 37°C for 24, 48, 72, and 96 h. All experiments were performed at least in triplicate.

### Cell Count

At the end of each incubation period, medium was removed by aspiration, and cells were detached by addition of 0.25% trypsin-EDTA for 3 min at 37°C. Cells were isolated, re-suspended, and counted using a Bürker-Türk hemocytometer. The mean number of cells for each ion concentration and the incubation period was expressed as percentage of the average number of cells in the negative controls for the corresponding ion and incubation period.

### Cellular Activity

Cellular activity was assessed with the 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT; Sigma-Aldrich). This test is based on the ability of living cells to reduce yellow MTT dye to insoluble purple MTT formazan crystals. At the end of each incubation period, the culture medium was replaced with culture medium supplemented with 0.5 µg/ml MTT and the cells in the 24-well microplates were incubated for an additional 3 h at 37°C. Liquid was then removed, 2-propanol was added, the plates were shaken for 5 min and the OD<sub>575</sub> was assessed in a microplate reader (Fluostar Optima; BMG Labtechnologies, Isogen Biosolutions bv, IJsselstein, NL). Results were expressed as a percentage

of the value obtained from the negative control dishes (set at 100%) for the corresponding ion and incubation period.

### RNA Isolation

Culture medium was removed from the cells, which were immediately lysed using the lysis buffer of an RNA isolation kit (InVisorb spin cell RNA mini kit; InVitec GmbH, Berlin, Germany). RNA was isolated according to the protocol of the manufacturer. Quantity and purity of the RNA were determined by 260/280 nm absorbance measurements. The RNA samples were subjected to RNase-free DNase digestion to remove any genomic DNA and to agarose gel electrophoresis to assess the intactness of RNA. Complete removal of genomic DNA was verified by polymerase chain reaction (PCR). RNA was reverse transcribed using the iScript kit according to the instructions of the manufacturer (Bio-Rad, Veenendaal, NL). The resulting cDNA was 25× diluted, aliquoted, and stored at –80°C.

### qPCR Assays

The real-time quantitative PCR (qPCR) assays were performed in triplicate with qPCR mastermix for SYBR<sup>®</sup> Green I (Abgene, Westburg, NL) in 96-well optical plates with primer concentrations of 0.8 µM. Primers for OPG, SOD, Catalase, and RPL13a were designed based on the gene sequences obtained from GeneBank using Perlprimer or Primer-3 software (Freeware and NCBI website, respectively), such that at least one primer from each set was spanning an exon–intron junction (Table 1). Primers for GAPDH were previously described by Emans et al.<sup>17</sup>; the sequences for the primers for RANKL were kindly provided by Dr. Holger Jahr (Erasmus University Medical Center, Rotterdam, NL). Plates were filled using a CAS-1200<sup>™</sup> pipetting robot (Corbett Life Science, Sydney, Australia). Five microliters of the diluted cDNA was used for each real-time qPCR reaction, which was performed in a MyIQ Real-time PCR apparatus (Bio-Rad Inc). PCR conditions were as follows: 15 min at 95°C, 40 cycles for 15 s at 95°C, and 15 s at 50–60°C (gradient), 15 s at 72°C, followed by a melt curve for OPG and RANKL, or 15 min at 95°C, 40 cycles for 15 s at 95°C, and 15 s at 60°C, 15 s at 72°C, followed by a melt curve for SOD and Catalase. Values were normalized to GAPDH (OPG and RANKL), or RPL13a (SOD and Catalase) and the control samples for each incubation time using the 2<sup>–ΔΔCt</sup> method.<sup>18</sup>

The ratio of OPG/RANKL was calculated by dividing the normalized fold expression of both genes within the same sample. Since the experiment was performed in triplicate and the PCR reactions were also performed in triplicate, the statistical analyses were performed on sets of 9 data.

### Oxidative Stress

Oxidative stress was assessed using three different assays. Firstly by assessment of glutathione (GSH) using a GSH-assay kit (APO/GSH kit; BioVision, ITK diagnostics, Uithoorn, NL). As positive controls in this assay cells were incubated for the respective follow-up periods with 100 µM H<sub>2</sub>O<sub>2</sub>, added daily. Additionally, oxidative stress was assessed by gene expression of both superoxide dismutase (SOD) and catalase, which was assessed using qPCR as described above. Primers are listed in Table 1.

### Statistical Analysis

All data were expressed as mean ± standard deviation. For the cell counts, three replicates were used, for the MTT tests,

**Table 1.** PCR Primers and Their Sequences Used for Real-Time PCR

Primer	Sequence (5'–3')	Amplicon Size (Nucleotides)	AT (°C)	Refs.
OPG-forward	GCAGCGGCACATTGGAC	69	60	Holger Jahr
OPG-reverse	CCCGGTAAGCTTTCCATCAA			
RANKL-forward	AGAGCGCAGATGGATCCTAA	180	56	
RANKL-reverse	TTCCTTTTGCACAGCTCCTT			
SOD-forward	ACAGCAGGCTGTACCAGTGC	102	60	17
SOD-reverse	CACATTGCCCCAAGTCTCCAA			
Catalase-forward	CGCAGAAAGCTGATGTCCTG	185	60	
Catalase-reverse	AAAGGCCCTGCTCCTTTAG			
Gapdh-forward	ACTTTGTGAAGCTCATTTCTGGTA	107	54	
Gapdh-reverse	GTGGTTTGAGGGCTCTTACTCCTT			
RPL13a-forward	GAGGTATGCTGCCCCACAAA	75	60	
RPL13a-reverse	GTGGGATGCCGTCAAACAC			

AT is optimal annealing temperature.

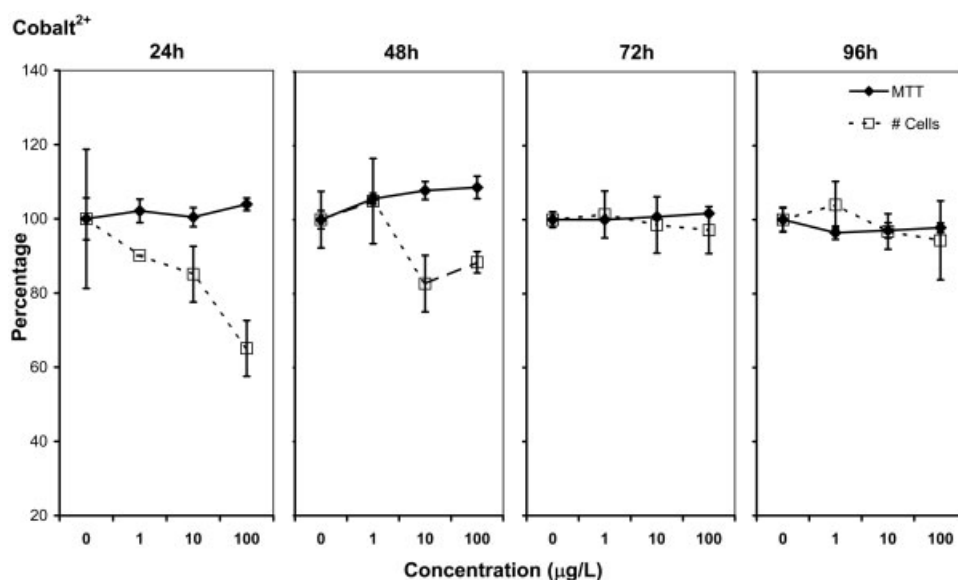
9 replicates were used, and for the OPG and RANKL PCR assays, also 9 replicates were used. For each ion group and incubation time period, statistically significant influences of concentration were tested by univariate analysis of variance (ANOVA), followed by a Bonferroni or Tukey–Kramer analysis for comparing individual groups. Probability less than 0.05 was adopted as the significance criterion. We used the Statistical Package for the Social Sciences version 15.0 for Windows (SPSS Inc., Chicago, IL).

## RESULTS

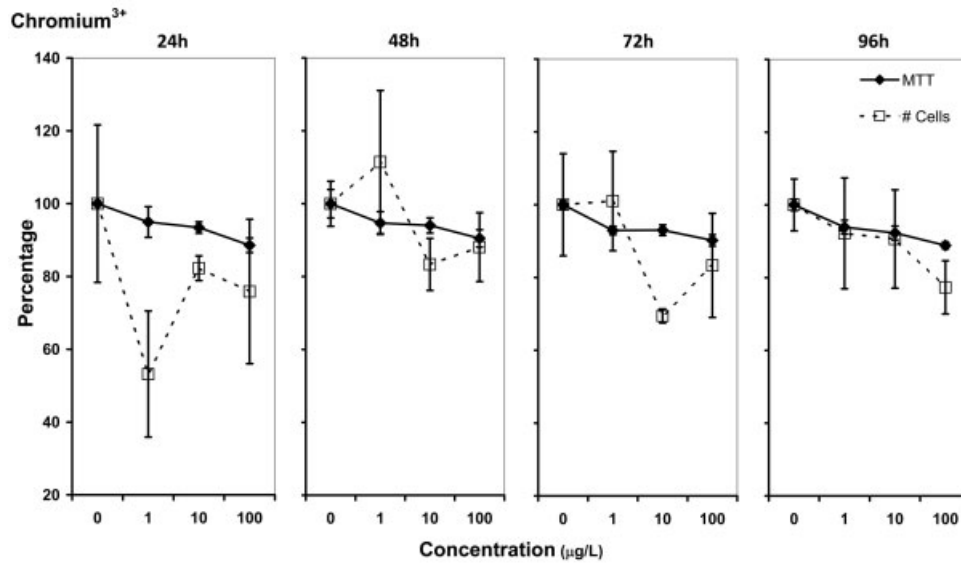
### Cell Count

In the presence of increasing concentrations of metal ions, the number of osteoblast-like cells decreased compared to the control samples (Figs. 1–3). Cobalt ions at

10  $\mu\text{g/L}$  reduced the number of osteoblast-like cells by  $17 \pm 8\%$  after 48 h ( $p = 0.001$ ). At 100  $\mu\text{g/L}$ , the reduction was  $12 \pm 3\%$  after 48 h ( $p = 0.010$ ). The strongest reduction was seen at 100  $\mu\text{g/L}$  after 24 h:  $35 \pm 8\%$  ( $p = 0.002$ ). Chromium ions decreased the number of osteoblast-like cells at 10  $\mu\text{g/L}$  after 48 and 72 h ( $17 \pm 7\%$ , resp.  $31 \pm 2\%$  decline;  $p < 0.001$ , resp.  $p = 0.001$ ). At 100  $\mu\text{g/L}$ , reductions of  $12 \pm 9\%$  ( $p = 0.041$ ) and  $23 \pm 7\%$  ( $p < 0.001$ ) were seen after 48 and 96 h, respectively. Cobalt and chromium combined at 1  $\mu\text{g/L}$  lowered cell numbers after 24 and 96 h ( $17 \pm 13$ , resp.  $13 \pm 4\%$ ;  $p < 0.001$ , resp.  $p < 0.001$ ). The 10  $\mu\text{g/L}$  concentration reduced cell numbers significantly after 24, 48, and 96 h ( $p < 0.001$ ,  $p = 0.041$ ,  $p = 0.002$ , resp.). These reductions varied from  $14 \pm 5\%$  to  $35 \pm 8\%$ .



**Figure 1.** Graph representing the number of Saos-2 cells as well as their mitochondrial activity after incubation with  $\text{Co}^{2+}$  ions at 1, 10, and 100  $\mu\text{g/L}$ . Incubation periods were 24, 48, 72, and 96 h. Data are expressed as mean ( $\pm$ SD) percentage relative to the control cell culture (without cobalt). Cell numbers were analyzed with Crystal Violet assays and mitochondrial activity with MTT assays.



**Figure 2.** Graph representing the number of Saos-2 cells as well as their mitochondrial activity after incubation with  $\text{Cr}^{3+}$  ions at 0, 1, 10, or 100  $\mu\text{g/L}$ . Incubation periods were 24, 48, 72, and 96 h. Data are expressed as mean ( $\pm$ SD) percentage relative to the control cell culture (without chromium). Cell numbers were analyzed with Crystal Violet assays and mitochondrial activity with MTT assays.

At 100  $\mu\text{g/L}$ , cobalt + chromium diminished cell numbers after 24, 48, 72, and 96 h (all  $p$ 's < 0.037).

#### Cellular Activity

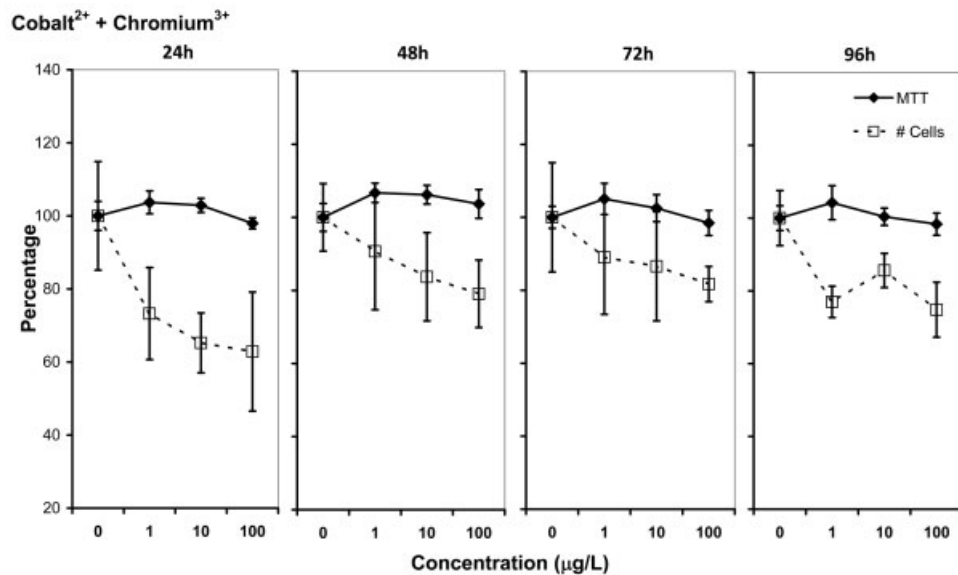
Cellular activity, as measured by the conversion of MTT, showed increased activity with increasing cobalt concentration at 48 h ( $p < 0.001$ ; see Figs. 1–3). Chromium however significantly reduced osteoblast activity at 1, 10, and 100  $\mu\text{g/L}$  at all incubation times (all  $p$ 's < 0.042). The strongest reduction ( $11 \pm 1\%$ ) was seen at 100  $\mu\text{g/L}$  after 96 h ( $p < 0.001$ ). Cobalt and

chromium combined increased osteoblast activity after 48 h at 1 and 10  $\mu\text{g/L}$  ( $p < 0.001$ , resp.  $p = 0.002$ ).

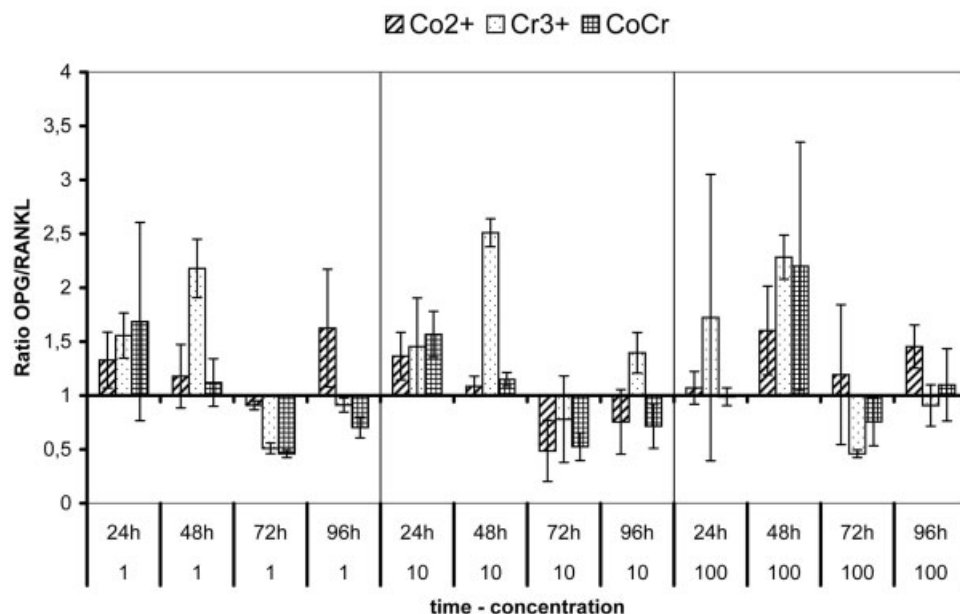
#### RANKL and OPG Expression

The expression of RANKL and OPG was expressed as the ratio of OPG:RANKL in Figure 4.

The highest ratios were observed when  $\text{Cr}^{3+}$  ions were present and with cobalt + chromium 100  $\mu\text{g/L}$  at 48 h. After 72 h of incubation, the ratio in almost all conditions was significantly lower ( $p < 0.05$ ). At 96 h,



**Figure 3.** Graph representing the number of Saos-2 cells as well as their mitochondrial activity after incubation with  $\text{Co}^{2+} + \text{Cr}^{3+}$  ions at 1, 10, or 100  $\mu\text{g/L}$ . Incubation periods were 24, 48, 72, and 96 h. Data are expressed as mean ( $\pm$ SD) percentage relative to the control cell culture (without  $\text{Co}^{2+} + \text{Cr}^{3+}$ ). Cell numbers were analyzed with Crystal Violet assays and mitochondrial activity with MTT assays.



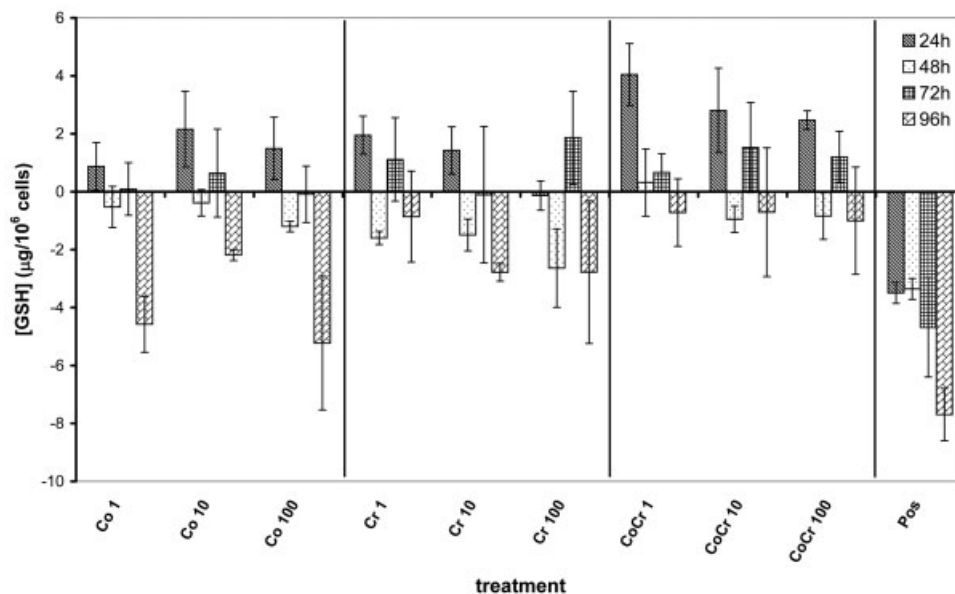
**Figure 4.** The ratio of mRNA levels of OPG and RANKL has been plotted after incubation with Co<sup>2+</sup>, Cr<sup>3+</sup>, or Co<sup>2+</sup> + Cr<sup>3+</sup> at 0, 1, 10, and 100 µg/L, indicated in the graph as Co1, Co10, and Co100 for Co<sup>2+</sup> at the different concentrations, Cr1, Cr10, Cr100 for Cr<sup>3+</sup> at the different concentrations and CoCr1, CoCr10, and CoCr100 for Co<sup>2+</sup> + Cr<sup>3+</sup> at the different concentrations. Incubation periods were 24, 48, 72, and 96 h. mRNA levels were assessed using qPCR and calculated using the Livak method.<sup>18</sup> The ratio of OPG mRNA/RANKL mRNA was calculated from three incubations which were measured in triplo (9 data points), then averaged and plotted relative to the control value at the given follow-up, which was set at 1. Significant differences are given in the text.

the ratio was as low or recovered somewhat; increasing ion concentrations did not seem to affect the ratio.

#### Oxidative Stress

Analysis of GSH levels in the cells revealed that GSH levels were reduced compared to the control (no additional ions added) in the presence of Co<sup>2+</sup> at 1, 10, and

100 µg/L at 96 h, in the presence of Cr<sup>3+</sup> ions at 1 µg/L at 48, at 10 µg/L at 48 and 96 h, and at 100 µg/L at 48 and 96 h. Co<sup>2+</sup> + Cr<sup>3+</sup> resulted in enhanced GSH concentrations at 1, 10, and 100 µg/L at 24 h. The positive control, incubation with H<sub>2</sub>O<sub>2</sub>, resulted in significant reduction of GSH concentration ( $p < 0.05$ ) at all follow-up moments (Fig. 5).



**Figure 5.** A histogram representing the changes in concentration of GSH, when Saos-2 cells were cultured in the presence of 0, 1, 10, or 100 µg/L of Co<sup>2+</sup>, Cr<sup>3+</sup>, or Co<sup>2+</sup> + Cr<sup>3+</sup> ions for 24, 48, 72, or 96 h. As a positive control Saos-2 cells were incubated with H<sub>2</sub>O<sub>2</sub> as described in the Methods Section. GSH concentration of 10<sup>6</sup> cells was assessed using an APO/GSH kit as described in the Methods Section. Significant differences are given in the text.

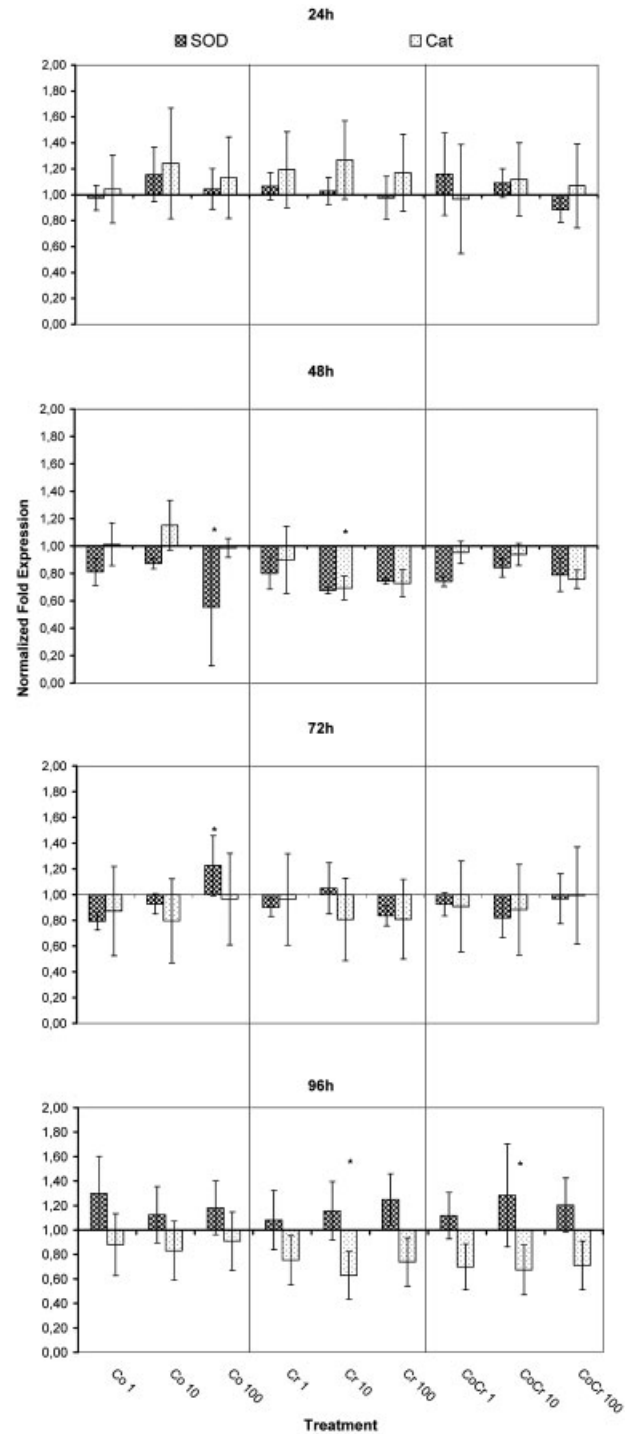
The expression of SOD and catalase was hardly changed after 24 h for all conditions. SOD expression was reduced with cobalt ions at 100  $\mu\text{g/L}$  at 48 h ( $p < 0.05$ ). Catalase expression was reduced with chromium ions at 10  $\mu\text{g/L}$  at 48 and 96 h and with cobalt + chromium ions at 10  $\mu\text{g/L}$  at 96 h ( $p < 0.05$ ). After 96 h, in all samples a definitive trend of oxidative stress response was observed: SOD expression levels were higher than the control, and the catalase expression levels lower (Fig. 6).

## DISCUSSION

We assessed the effects of cobalt and chromium ions on human osteoblast-like cells in relation to cytotoxicity (cell number and cell activity). We also determined the effects on these ions on the expression of bone turnover regulatory proteins RANKL and OPG, as well as on the oxidative stress response. We found that higher ion concentrations were more cytotoxic. Chromium was more toxic than cobalt, yet both ions combined yielded the greatest cell reduction. Cell activity showed a positive relationship to chromium ion levels, thus it decreased when the chromium ion concentration increased. It is remarkable that the cytotoxic effects of the metal ions on cell count, cobalt especially, were strongest in the first 24 h. It appears that there is some form of adaptive response, for which we have no clear explanation. Sensitization of cells to stimuli is a common phenomenon in cell cultures. In such cases toxicity will only show in the long term. Others<sup>5</sup> also found time (and dose) dependent decreases of osteoblast numbers after addition of cobalt and chromium ions, but used much higher ion concentrations (0–10 ppm, i.e., 0–10,000  $\mu\text{g/L}$ , resp. 0–150 ppm, i.e., 150,000  $\mu\text{g/L}$ ). Our results, with clinically relevant concentrations, could indicate that long-term cobalt and chromium ion exposures are not necessarily more harmful than short-term exposure.

Cell activity decreased in a dose-dependent manner after chromium ion addition, but showed no temporal dependance. After exposure to  $\text{Co}^{2+}$ , activity increased in the first 48 h, then remained fairly stable. Fleury et al.<sup>5</sup> found reduced activity with both  $\text{Cr}^{3+}$  and  $\text{Co}^{2+}$ , but used 100 times higher concentrations. It is plausible that during the first 24–48 h after cobalt addition, the osteoblasts up-regulate their activity in an attempt to try to balance their reduction in cell numbers, but plateau after 48 h.

The oxidative stress assays gave results that were somewhat contradictory. Significant reductions in GSH concentrations were observed after 96 h with all concentrations of  $\text{Co}^{2+}$  and at 10 and 100  $\mu\text{g/L}$  of  $\text{Cr}^{3+}$ , which are in the order of the positive control. The combination of both ions did not reveal any reduction when compared to the control samples. Nevertheless, there were significantly higher levels of GSH in these samples at 24 h follow-up. So after 24 h also these samples result in a dose-dependent decrease in GSH. The data suggests that cells, in an attempt to protect



**Figure 6.** Graph representing the expression of superoxide dismutase (SOD) and catalase (Cat) of Saos-2 cells incubated with 0, 1, 10, or 100  $\mu\text{g/L}$  of  $\text{Co}^{2+}$ ,  $\text{Cr}^{3+}$ , or  $\text{Co}^{2+} + \text{Cr}^{3+}$  ions (indicated in the graph as Co1, Co10, Co100, Cr1, Cr10, Cr100, CoCr1, CoCr10, and CoCr100) for 24, 48, 72, or 96 h. mRNA levels were measured using qPCR and calculated with the Livak method<sup>18</sup> as described in the Methods Section. Significant differences are given in the text.

themselves, began to enhance GSH levels when both ions were added. To our knowledge this has never been described before and warrants further investigation. In contrast, the SOD and catalase mRNA expressions levels were not dependent on the ion dosages of

$\text{Co}^{2+}$ ,  $\text{Cr}^{3+}$ , or  $\text{Co}^{2+} + \text{Cr}^{3+}$ . For this we do not have an explanation, other than perhaps SOD and catalase gene expression in these studies were not very much affected by  $\text{Co}^{2+}$  and  $\text{Cr}^{3+}$  ions, and apparently do not play a major role in protection of the cells. Furthermore, we found that the OPG to RANKL ratio decreased after 72 h in almost all conditions, indicating net bone loss. This coincided with an oxidative stress response after 96 h in all samples.

Although our experiment is an *in vitro* study, the tested ion concentrations are clinically relevant. In our own 10-year follow-up study of the cemented Stanmore metal-on-metal total hip prosthesis, serum cobalt and chromium ion concentrations were found ranging from 0.5 to 11 and 0.5–9.5  $\mu\text{g/L}$  respectively.<sup>16</sup> A study on failed metal-on-metal bearings showed that there is a very strong correlation between serum cobalt and chromium ion concentration and joint fluid cobalt and chromium ion concentration; the latter was found to be 37 (cobalt) or 47 (chromium) times larger than the serum concentration.<sup>19</sup> This implies that the actual ion concentrations in proximity to the patient's acetabulum and femur could vary between 19 and 447  $\mu\text{g/L}$ .

Our results support available studies describing the detrimental influence of cobalt and chromium ions on osteoblasts (see Ref. 20 for a short review). Osteoblasts exposed to these ions undergo a dose-dependent reduction in proliferation, show reduced alkaline phosphatase activity, induce oxidation and nitration of proteins and disorganization of the expression of antioxidant enzymes,<sup>5</sup> release pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor (TNF)- $\alpha$  and inhibited osteocalcin release and collagen type-I synthesis.<sup>1</sup> On the other hand, Fu et al.<sup>21</sup> found no significant cytotoxicity of  $\text{Cr}^{3+}$  in rat calvarial osteoblasts and cobalt and chromium ion addition to MG-63 osteoblast-like cells was not associated with IL-6 or TNF- $\alpha$  secretion.<sup>22</sup> Chromium particles however did induce IL-6 synthesis in MG-63 osteoblasts, which in turn may induce osteoclast differentiation.<sup>23</sup> Although different model systems were used in these studies, overall it can be stated that osteoblast function is affected by cobalt and chromium ions and pro-inflammatory cytokines seem to play a major role in this process.

It is not known however, whether bone turnover regulatory proteins RANKL and OPG are involved in the effects of cobalt and chromium ions on osteoblast and osteoclast functioning. We therefore assessed whether cobalt and chromium ions affect RANKL and OPG. Our results show increased OPG to RANKL ratio's 48 h after addition of  $\text{Cr}^{3+}$ , followed by reduced ratio's at 72 h and beyond. Although there was no clear effect of concentration, there was late net bone loss at Co 10  $\mu\text{g/L}$ , Cr 1  $\mu\text{g/L}$  and higher, and at Co + Cr 1  $\mu\text{g/L}$  and higher. For clinical practice, this suggests that even in well functioning metal-on-metal implants with systemic cobalt and chromium levels around 1  $\mu\text{g/L}$ , local peri-prosthetic osteolytic reactions

may be expected. The oxidative stress response visible after 96 h further strengthens this assumption. On the other hand, high (100  $\mu\text{g/L}$ ) ion concentrations were not more osteolytic than low (1  $\mu\text{g/L}$ ) concentrations based on the OPG to RANKL ratios.

Certain limitations apply to our study. We found relatively large standard deviations, especially in the cell count data in the first 24 h. This is possibly due to the relatively low number of seeded cells in order to culture up to 96 h, and a low number of replicates. Secondly, we have only studied osteoblast-like cells since these are more easily cultured than osteoclasts. The osteoblasts were studied *in vitro* and therefore their natural synovial environment as found in a joint replacement patient was excluded. It is possible that cobalt and chromium ions not only affect osteoblast RANKL, but also lead to TNF- $\alpha$  release<sup>24</sup> and hence RANKL expression. TNF- $\alpha$  and IL-6 expression was not measured in our study. Furthermore, we have excluded lymphocytes in our experiment. Lymphocytic reactions have been found in retrieved periprosthetic tissues from metal-on-metal arthroplasties<sup>25</sup>; lymphocytes can also lead to RANKL activation.<sup>26</sup> Tissues retrieved from metal-on-metal arthroplasties showed lower amounts of multinucleated giant cells than expected from metal-on-polyethylene revisions.<sup>27</sup> This suggests that giant cells may play a less important role in peri-prosthetic loosening and failure of metal-on-metal bearings. In contrast, the influence of lymphocytes on RANKL and OPG and their role in metal-on-metal prosthetic failure still requires elucidation.

In conclusion, the main findings of our *in vitro* study are that cobalt and chromium ions reduce human osteoblast activity, reduce the OPG to RANKL ratio suggesting osteolysis and lead to oxidative stress.

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